

**Amendments to the Specification:**

Please amend the specification as follows:

Please replace paragraph starting at page 1, line 4, with the following rewritten paragraph:

This application is a Divisional application of Serial No. 08/340,664 filed November 16, 1994, which is a ~~This is a~~ Divisional application of prior application Serial No. 08/087,471, filed on July 2, 1993, which is a File Wrapper Continuation of Serial No. 07/821,478, filed on January 15, 1992, which is a Continuation of Serial No. 07/404,970, filed on September 8, 1989, now abandoned, which is a Continuation-In-Part of Serial No. 07/393,851, filed on August 14, 1989, which issued as U.S. Patent No. 5,010,010 on April 23, 1991, which application, in turn, is a File Wrapper Continuation of Serial No. 06/921,684, filed on October 22, 1986, abandoned.

Please replace paragraph starting at page 6, line 16, with the following rewritten paragraph:

Employing the promoter and signal sequence of Staphylococcus aureus protein A we have expressed hPTH in ~~Eaeherichis~~ Escherichia coli as a secretory peptide. Immunoreactive PTH was isolated both from growth medium and periplasmic space. We obtained up to 10 mg/l hPTH as judged by reactivity in radioimmunoassay.

Please replace paragraph starting at page 7, line 12, with the following rewritten paragraph:

hPTH immunoreactive material was concentrated from the growth medium by passage through a S Sepharose Fast flow column and eluted quantitatively. Recombinant hPTH was purified by reverse phase HPLC. The column was eluted with a linear gradient of acetonitrile/trifluoroacetic acid. A major peak (fractions 32 and 33) with the same retention time as standard hPTH(1-84) was resolved into two peaks in a second HPLC ~~urification~~ purification step. The major peak from the 2.HPLC eluted exactly as standard hPTH(1-84) and co-chromatographed with hPTH(1-84) as one symmetric peak. SDS-PAGE of the peak fraction showed one band co-migrating with hPTH standard suggesting that the recombinant PTH was essentially pure. The recombinant hPTH was subjected to N-terminal amino acid analysis. We were able to determine unambiguously 45 amino acids from the N-terminal end

in the *E. coli* protein and 19 amino acids in the yeast protein. The sequence was identical to the known sequence of hPTH. The sequence analysis indicated that the recombinant PTH was more than 90 percent pure. The recombinant hPTH from *E. coli* and *Saccharomyces cerevisiae* was fully active in adenylate cyclase assay and also induced hypercalcemia in rats after injection

Please replace paragraph starting at page 8, line 25, with the following rewritten paragraph:

Figure 8...In panels a-c illustrates the analysis ~~Analysis~~ of expression products by SDS—PAGE and immunoblotting.

*Saccharornycs cerevisiae* transformed with a Pm cDNA carrying plasmid was grown in liquid culture medium. The secreted products were concentrated and analyzed on SDS—PAGE. Panel a shown a silver stained gel with molecular size marker (lane 5), hPTH standard (lane P), and concentrated yeast growth medium (lane 1). After blotting onto a PVDF membrane, blots were probed with hPTH specific antibodies, one reactive against the aminoterminal part of the hormone (panel b), another reactive against the middle region of the hormone (panel c). Lanes in panel b and c are numbered as in panel a.

Please replace paragraph starting at page 9, line 28, with the following rewritten paragraph:

Figure 13. Purity of purified hPTH (1-84,Q26). Yeast growth medium from yeast strain BJ1991 transformed with the expression plasmids pαUXPTH-Q26 were concentrated and purified by reversed phase HPLC as described in Experimental Protocol. The purity of the recombinant hormone was then analyzed by analytical HPLC (Panel A) and SDS PAGE (Panel B, lane 2). In Panel B the purified hPTH (1-84,Q36) is compared with the wild type hormone purified by two runs on HPLC (lane 3). The molecular weight ~~market~~ marker in lane M is the same as in Figure 2. Lane 1 shows a reference PTH produced in *E. coli*.

Please replace paragraph starting at page 11, line 3, with the following rewritten paragraph:

The invention further provides methods of producing and isolating the plasmids and transformed microorganisms. Poly(A) selected RNA was isolated from human parathyroid adenomas collected immediately after surgery. The poly(A) RNA was enriched for correct

size mRNA by ultracentrifugation through sucrose gradients. Preproparathyroid hormone of correct molecular weight was translated in vitro from this size fractionated poly(A) RNA as judged by sodium dodecylsulphate polyacrylamide gel electrophoreses after immuno precipitation with antiparathyroid antiserum. The specific messenger RNA for the human PTH was used as template for complementary DNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase. After removal of the RNA templates by alkali hydrolysis, the second strand complementary DNA was synthesized by incubating the purified first strand DNA in the presence of the Klenow fragment of E. coli DNA polymerase I. The double stranded ~~complementary~~ complementary DNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1. and complementary DNA longer than 500 base pairs was isolated after neutral sucrose gradient centrifugation. Approximately 20 bases long d(C)-tail protrusions were enzymatically added to the 3' ends of the cDNA. This modified complementary DNA was annealed to restriction endonuclease PstI cleaved and d(G)-tailed vector pBR322. Resulting recombinant plasmid DNA's were transformed into E. coli KI2 BJ 5183. Positive transformants were analyzed for by colony hybridization using two different synthetic ~~deoxyribo~~oligonucleotides oligodeoxyribonucleotides which covered the N-terminal coding region as well as the 3' non-coding part of the hormone mRNA sequence, respectively. Six out of 66 clones that were positive for both probes were submitted for detailed analysis by restriction endonuclease mapping showing that they all were identical except for some size heterogeneity at the regions flanking the start codon and the XbaI site 3' for the stop codon. One clone, pSShPTH-10, was subjected to DNA sequence analysis revealing a 432 nucleotide long human parathyroid hormone complementary DNA sequence inserted in the PstI site of pBR 322. The entire cDNA sequence was found to be identical to the sequence previously described by Hendy, et al., supra, except for a 5 base pair deletion in front of the start codon.

Please replace paragraph starting at page 16, line 17, with the following rewritten paragraph:

hPTH is an easily degraded polypeptide. Already in the parathyroid gland large amounts of carboxyl-terminal PTH fragments are generated.<sup>1/</sup> Structural studies have suggested that hPTH may contain two domains with the easily cleaved region placed in a connecting stalk between these domains.<sup>5/</sup> Not surprisingly therefore, degradation of hPTH

has been a major problem when the hormone is expressed in heterologous organisms. In *E. coli* low expression levels combined with degraded hormone peptides of short half-life were observed.<sup>6-8/</sup> The most successful expression system for hPTH so far is *Saccharomyces cerevisiae* where the hormone is expressed as a secretory peptide.<sup>9/</sup> By that approach we were able to obtain significant amounts of authentic hPTH(1-84) with full biological activity. But even if conditions were found which eliminated proteolytic attacks at some sites in the putative stalk region of the hormone, a significant fraction of the secreted peptides was still cleaved after a pair of basic amino acids found in the hPTH sequence reducing the yield of full length peptide hormone. The cleavage site resembles that recognized by the yscF protease (the KEX2 gene product).<sup>10,11/</sup> We reasoned that the elimination of the putative yscF cleavage in hPTH could lead to a significant gain in the yield of undegraded hPTH secreted from yeast. In the present report we describe the removal of the putative yscf cleavage sites by *in vitro mutagenesis* of the hPTH coding region. When the amino acid at position 26 in hPTH was changed from Lysine (K26) to Glutamine (Q26), the major degradation product hPTH(27-84) previously observed disappeared in the growth medium and the yield of full-length hormone increased 5- to 10-fold. The secreted degradation resistant ~~hPTH(1-84, Q26)~~ hPTH(1-84, Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence. Furthermore, the introduced mutation had no effect on the biological activity of the hormone as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

Please replace paragraph starting at page 19, line 18, with the following rewritten paragraph:

The yeast strain BJ1991 was transformed with the plasmids paUXPTH-Q26 containing the mutated hPTH coding region. One transformant was grown in YNBGC medium lacking uracil and the cell free medium was concentrated and analyzed in different gel systems. Figure 12 shows a silver-stained SDS polyacrylamide gel where concentrated medium from paUXPTH-Q26 transformed cells (mutated hPTH, lane 1) is compared with that from paUXPTH-Q26 transformed cells (wild type hPTH, lane 2). In the latter case the strongest band has a molecular mass lower than the standard hPTH, and previous microsequencing has shown that it corresponds to the hormone fragment hPTH(27-84). In the lane with the mutated product (lane 1), this band is absent showing that the cleavage

between amino ~~acid~~ acids 26 and 27 has been totally eliminated as a result of the mutation. Now the major product is a polypeptide that migrates close to the full length hPTH standard. Consistently, this band had a migration slightly faster than the standard in an anionic gel system and a migration slightly slower than the standard in a cationic gel system in accordance with the single charge difference between the mutated (one positive charge less) and the wild type hormone. In addition to the main product a few weaker bands were present of apparently higher molecular mass which might be O-glycosylated forms of the hormone.

Please replace paragraph starting at page 20, line 26, with the following rewritten paragraph:

Since the elimination of the internal cleavage of the secreted hPTH leads to fewer polypeptides with similar properties in the growth medium, this form of the hormone could also be isolated by a simplified purification procedure. Already in the first concentration step using a Sepharose S column, a certain purification is achieved. All hPTH immunoreactive material is retained, but some high molecular weight material is removed in the pH6 wash of the Sepharose S column. This first concentrated eluate already contained more than 80 percent hPTH(1-84, Q26). Then, a single run on a ~~reversed~~ reverse phase HPLC C18 column, was enough to give near homogeneous hPTH(1-84, Q26). The purity was checked both by SDS polyacrylamide gelelectrophoresis and sensitive silver-staining, and by analytical HPLC as illustrated in Figure 13A. A single peak was found in the chromatogram (Figure 13A), and a single band with only a trace of a closely migrating hPTH band (probably an O-glycosylated form of the hormone) could be seen in the SDS polyacrylamide gel (Figure 13B). When the yield of pure full length mutated hormone was compared with that of the wild type, 5 to 10 fold higher yields were usually achieved. This is consistent with our previous estimate of the fraction of full length hormone (up to 20 percent) obtained when the wild type is expressed.<sup>9/</sup>

Please replace paragraph starting at page 29, line 21, with the following rewritten paragraph:

The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20 g/ml tRNA and 0.05% sodium ~~pyrophosphate~~ pyrophosphate) supplemented with 32P-labelled DNA probe. (Woods supra).

Please replace paragraph starting at page 29, line 26, with the following rewritten paragraph:

The DNA used as a hybridization probe was one of two different synthetic ~~deoxyribo-~~  
~~oligonucleotides~~ oligodeoxyribonucleotides of which the sequences were deduced from the published human PTH cDNA sequence of Hendy, supra. The first probe was a 24-mer oligonucleotide originating from the start codon region of the human preproPTH coding sequence having a nucleotide sequence reading TACTATGGACGTTTTCTGTACCGA. The second oligonucleotide was a 24-mer spanning over a cleavage site for the restriction endonuclease XbaI located 31 nucleotides downstream of the termination codon and consisted of the nucleotide sequence CTCAAGACGAGATCTGTCACATCC.

Please replace paragraph starting at page 30, line 5, with the following rewritten paragraph:

The hybridized filters were washed in 6xSSC, 0.05% sodium pyrophosphate at 42 degrees Centigrade prior to autoradiography. Sixty-six clones were found to be positive for both probes as judged from hybridization to both copies of the duplicate replica filters. All those were picked from the original filters with the stored cDNA library and amplified for ~~indefinitive~~ indefinite storage at -70 degrees Centigrade. Six of these were chosen for plasmid preparation and a more detailed analysis by restriction endonuclease mapping, showing that all were identical except for some size ~~heterogeneity~~ heterogeneity at the regions flanking the start codon and Xba I site, respectively.

Please replace paragraph starting at page 30, line 38, with the following rewritten paragraph:

Before the hPTH-yeast-expression project was initiated, a family of general yeast expression vectors were developed. One of these, pL4, later was used to make pSS $\alpha$  LX5-hPTH1, as described below:

Please replace paragraph starting at page 34, line 6, with the following rewritten paragraph:

The yeast strain FL200 ( $\alpha$ , ura3, leu2) was transformed with the plasmids p $\alpha$ LX5 and pSS $\alpha$  LX5-hPTH1 using the spheroplast method. One transformant of each kind was grown up in leu<sup>-</sup> medium and aliquots of the cell-free medium were analyzed by SDS-PAGE developed by silver-staining. Two major bands were seen in the medium from the pSS $\alpha$

LX5-H1 transformant that were not present in the medium from the p LX5 transformant: one band of approximately 9000 daltons, the expected size of HPTH, and one band of approximately 16000 daltons that could correspond to an unprocessed MF $\alpha$ 1-hPTH fusion product. Both polypeptides reacted with antibodies against human PTH in a manner identical to the native hormone.

Please replace paragraph starting at page 36, line 28, with the following rewritten paragraph:

In order to change the amino acid at position 26 in the human PTH from lysine to glutamine, the fusion gene in *pa*PTH-M13- $\Delta$ EA was further modified by in vitro mutagenesis using the "Muta~gene™ in vitro mutagenesis kit" obtained from Bio-Rad based on the method of Kunkel; Kunkel, T.A., Roberts, J.D., and Sakour, R.A. (1987) "Rapid and efficient site-specific mutagenesis without phenotypic selection" in Methods of Enzymology, Enzymology, (Wu, R., and Grossman, L., eds.) vol. 154, pp 367-381, which is hereby incorporated by reference. The E. coli strain or CJ236 (*dut*, *ung*, *thi*, *rel* A; pCJ105 (Cm<sup>r</sup>)) was transformed with the *pa*PTH-M13- $\Delta$ EA plasmid. The single-stranded DNA that was prepared from the phage contained a number of uracils in thymine positions as a result of the dut mutation (inactivates dUTPase) and the ung mutation (inactivates the repair enzyme uracil N- glycosylase). An oligonucleotide with the sequence GGCTGCGTCAGAAGCTGC was made where all nucleotides except the ninth are complementary to an internal PTH sequence in *pa*PTHx-M13. When this oligonucleotide was annealed to the single-stranded DNA, the following heteroduplex was generated: